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Evaluation of *in vitro* vs. *in vivo* methods for assessment of dermal absorption of organic flame retardants: A review

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Abstract

There is a growing interest to study human dermal exposure to a large number of chemicals, whether in the indoor or outdoor environment. Such studies are essential to predict the systemic exposure to xenobiotic chemicals for risk assessment purposes and to comply with various regulatory guidelines. However, very little is currently known about human dermal exposure to persistent organic pollutants. While recent pharmacokinetic studies have highlighted the importance of dermal contact as a pathway of human exposure to brominated flame retardants, risk assessment studies had to apply assumed values for percutaneous penetration of various flame retardants (FRs) due to complete absence of specific experimental data on their human dermal bioavailability. Therefore, this article discusses the current state-of-knowledge on the significance of dermal contact as a pathway of human exposure to FRs. The available literature on *in vivo* and *in vitro* methods for assessment of dermal absorption of FRs in human and laboratory animals is critically reviewed. Finally, a novel approach for studying human dermal absorption of FRs using *in vitro* three-dimensional (3D) human skin equivalent models is presented and the challenges facing future dermal absorption studies on FRs are highlighted.

Keywords

Flame retardants, dermal absorption, human exposure, human skin equivalents, bioavailability.

47

List of Acronyms

BFRs	brominated flame retardants
BPA	bisphenol A
BTBPE	1,2-bis(2,4,6 tribromophenoxy)ethane
DBDPE	Decabromodiphenylethane
EU	European Union
EVCAM	european centre for validation of alternative methods
FRs	flame retardants
FT	full-thickness skin
HBCD	hexabromocyclododecane
HSE	human skin equivalent
KC	keratinocytes
K_{OW}	octanol/water partition coefficient
LC	langerhans cells
NBFRs	novel brominated flame retardants
OATP	organic anion transporting polypeptides
OECD	organisation for economic co-operation and development
PA	percutaneous absorption
PBDEs	polybrominated diphenyl ethers
PBT	persistent, bioaccumulative and toxic
PCBs	polychlorinated biphenyls
PFRs	organophosphate flame retardants
PK	pharmacokinetic
POPs	persistent organic pollutants
RDP	resorcinol bis-diphenylphosphate
SC	stratum corneum
RHE	reconstructed human epidermis
TBB	2-ethylhexyl 2,3,4,5-tetrabromobenzoate
TBBPA	tetrabromobisphenol A
TBPH	Bis(2-ethylhexyl)tetrabromophthalate
TCEP	tris(2-chloroethyl) phosphate
TCIPP	tris(2-chloro-1-methylethyl) phosphate
TDCPP	tris(1,3-dichloro-2-propyl) phosphate
TRIS	tris (dibromopropyl) phosphate

USEPA	United States environment protection agency

48

Introduction

Organic flame retardants (FRs) are a diverse group of chemicals used to prevent or reduce the flammability and combustibility of polymers and textiles. The major members of this group are polybrominated diphenyl ethers (PBDEs), hexabromocyclododecane (HBCD), tetrabromobisphenol A (TBBP-A), novel brominated flame retardants (NBFRs), as well as organophosphate flame retardants (PFRs) (Ghosh, et al. 2011; van der Veen and de Boer 2012).

Although polychlorinated biphenyls (PCBs) were mainly applied as heat transfer fluids in electric equipment, capacitors and transformers, one of their major advantages as heat transfer fluids was flame-retardancy. Thus, PCBs were highly desirable for applications where fire was a threat to life and property, such as in electrical equipment in commercial buildings, hospitals, in hydraulic systems in foundries, and in heat transfer systems. Furthermore, PCBs were also applied to flame-proof polyimide (nylon-type) and polyolefin yarns. Due to their persistent, bioaccumulative and toxic (PBT) properties, the production and usage of PCBs were banned throughout most of the industrialized world in the 1970s (Erickson and Kaley 2011; Fiedler 2001).

PBDEs have found wide application as FRs for plastics, textiles, electronics casings and circuitry. The fully brominated product (DecaBDE) dominated worldwide production with a global demand of 56,100 t in 2001, compared to 7,500 and 3,790 t for the less brominated PentaBDE and OctaBDE formulations, respectively (BSEF 2013). In 2001, the world market demand for HBCD was 16,700 tons, 57% of which was in Europe (Covaci, et al. 2006). The principal application of HBCD is in expanded and extruded polystyrene foams used for building insulation , but it has also been used to flame retard textiles and housing for electrical items (KEMI (National Chemicals Inspectorate) 2008). TBBP-A is the most widely used BFR with a production volume of 170,000 tons in 2004, applied mainly for epoxy resins

used in printed circuit boards of electric and electronic equipments (Covaci, et al. 2009a). As PBDEs, HBCD, and ~20% of the production of TBBP-A are blended physically within (and referred to as “additive” FRs) rather than bound chemically (and known as “reactive” FRs) to polymeric materials; they migrate from products, following which their persistence and bioaccumulative character leads to contamination of the environment including humans (Harrad, et al. 2010a). This is of concern owing to their potential environmental and toxicological risks including: endocrine disruption, neurodevelopmental and behavioural disorders, hepatotoxicity and possibly cancer (Darnerud 2008; Hakk 2010; Wikoff and Birnbaum 2011). Moreover, the few data available from human epidemiological studies imply effects on: male reproductive hormones (Johnson, et al. 2013; Meeker, et al. 2009), semen quality (Akutsu, et al. 2008), thyroid hormone homeostasis (Turyk, et al. 2008), cryptorchidism (Main, et al. 2007), hormone levels and fecundability in adult women (Harley, et al. 2010), as well as lower birth weight and length (Chao, et al. 2007; Lignell, et al. 2013). Such evidence has contributed to complete EU bans for the Penta- and Octa-BDE formulations, and restrictions on the use of Deca-BDE (Roberts, et al. 2012). In addition, PBDEs associated with Penta- and Octa-BDE are listed under the UNEP Stockholm Convention on POPs, while Deca-BDE is currently under consideration for listing under Annexes A, B and/or C of the convention (Stockholm convention on POPs 2009). Furthermore, HBCD will be phased out following its recent listing under Annex A of the Stockholm Convention (Stockholm convention on POPs 2013). Despite such restrictions on their production and use, human exposure to PBDEs and HBCD is likely to continue for some time, given the ubiquity of flame retarded products remaining in use and entering the waste stream, coupled with the environmental persistence of these BFRs (Harrad and Diamond 2006).

These restrictions on the use of PBDEs and HBCD have paved the way for the use of NBFRs as replacements with an estimated global production volume of 100,000 tonnes in 2009 (Harrad and Abdallah 2011). Major NBFRs are: DBDPE (Decabromodiphenylethane), BTBPE (1,2-bis(2,4,6 tribromophenoxy)ethane), TBB (2-ethylhexyl 2,3,4,5-tetrabromobenzoate), and TBPH (Bis(2-ethylhexyl)tetrabromophthalate) (further details are provided in Table SI-1). While information regarding the environmental occurrence of several NBFRs has become available recently (Covaci, et al. 2011), very little is known about their toxicological properties and the pathways and magnitude of human exposure to these chemicals. Nevertheless, several NBFRs bear striking structural similarity to PBDEs (e.g. DBDPE is a very close analogue of BDE-209) and are reported to have similarly low vapour pressures and water solubilities, as well as high K_{OW} values, and PBT characteristics (Covaci, et al. 2011; Harrad and Abdallah 2011).

In addition to BFRs, PFRs have been associated with a wide range of applications (Table SI-1). Likely linked to the aforementioned restrictions on PBDEs, EU market demand for PFRs increased from 83,700 tons in 2004 to 91,000 tons in 2006 (EFRA 2007). Tris(2-chloroethyl) phosphate (TCEP), tris(2-chloro-1-methylethyl) phosphate (TCIPP) and tris(1,3-dichloro-2-propyl) phosphate (TDCPP) were all subject to an EU risk assessment process under an Existing Substances Regulation (EEC 793/93) (Regnery and Puttmann 2010). Despite less stability and overall environmental persistence than PBDEs, they were classified as persistent organic compounds in the aquatic environment and reported to fulfil PBT criteria. In addition, several studies have reported them to display adverse effects including reproductive toxicity and carcinogenic effects on lab animals (Regnery, et al. 2011). Hence TCEP is classified by the EU as a “potential human carcinogen” (Regnery and Puttmann 2010), while TDCPP is classified under regulation EC 1272/2008 as a category 2 carcinogen (ECHA 2010).

Human exposure to FRs. Several studies have reported on levels of different FRs in various environmental and human matrices (Covaci, et al. 2011; Covaci, et al. 2009b; Harrad, et al. 2010b; Law, et al. 2014; van der Veen and de Boer 2012). Current understanding is that non-occupational human exposure to BFRs occurs mainly via a combination of diet, ingestion of indoor dust, dermal contact with dust/consumer products, and inhalation of indoor air (Figure 1) (Abdallah, et al. 2008a; Frederiksen, et al. 2009; Watkins, et al. 2011). The exact contribution of these pathways varies substantially between chemicals, between individuals according to lifestyle, and is further complicated by international variations in FR use (Abdallah and Harrad 2009; Abdallah, et al. 2008a; Abdallah, et al. 2008b; Harrad, et al. 2008b). While it is established that the main exposure route to several POPs (e.g. PCBs and DDT) is through diet, studies from North America report indoor dust (via ingestion or dermal contact) as the major exposure pathway for all age groups to PBDEs contributing 70-80% to the average overall daily exposure (Lorber 2008; Trudel, et al. 2011). Elsewhere, while dust ingestion appears particularly important for toddlers and young children; other exposure pathways make substantial contributions to the overall adult intake of BFRs (Abdallah, et al. 2008a; Harrad, et al. 2010b; Harrad, et al. 2008a; Roosens, et al. 2009). In contrast to PBDEs, only a few studies are available that address human exposure to NBFRs and PFRs (Ali, et al. 2012; Covaci, et al. 2011; Stapleton, et al. 2011). Currently very little is known about dermal exposure as a route of human exposure to FRs in indoor dust or FR-treated products. This paucity of information was evident in the EU risk assessment reports on TBBPA (EU Risk Assessment Report 2006) and BDE-209 (EU Risk Assessment Report 2002) where the lack of experimental data has led to the assumption of dermal absorption efficiencies based on consideration of compound-specific physicochemical properties and extrapolation from data available for PCBs. Furthermore, several authors have discussed the absence of experimental

148 data on dermal absorption of various FRs and highlighted the potential inaccuracies of
149 current estimates of human exposure to these FRs owing to a general lack of knowledge on
150 the percutaneous route (Boyce, et al. 2009; Garner, et al. 2006; Trudel, et al. 2011; U.S. EPA
151 1992). Therefore, the lack of experimental information on human dermal uptake of FRs from
152 dust and source materials, represents an important research gap that hampers accurate
153 assessment of human exposure to FRs. However, efforts to fill this gap are hindered by
154 several difficulties including: ethical issues encountered with human studies, inter-species
155 variation in dermal structure and uptake that cast doubt on the accuracy of extrapolation or
156 allometric scaling of animal data to humans, and tighter regulations on *in vivo* tests involving
157 animals.
158 Against this backdrop, this paper: *(a)* provides a critical review of the current state-of-
159 knowledge on dermal absorption of FRs, *(b)* discusses the paradigm shift in toxicity testing
160 from *in vivo* to *in vitro* dermal bioavailability studies and *(c)* suggests effective novel
161 approaches to studying human dermal uptake of FRs, with special emphasis on *in vitro* 3D
162 human skin percutaneous assays, that are finding increasing application in the pharmaceutical
163 and cosmetics sectors (Gibbs, et al. 2013; Kandarova, et al. 2013; Tornier, et al. 2010).
164
165 **Skin as a barrier for systemic exposure to xenobiotic chemicals.**
166 Skin is the largest body organ, with a surface area of ~ 2 m^2 and weighing about 5 kg in adult
167 humans (Godin and Touitou 2007). This multi-layered organ acts mainly to protect the body
168 from the surrounding environment, thus froming an efficient permeation barrier for
169 exogenous molecules. Human skin is formed of 3 main layers, namely: epidermis, dermis and
170 hypodermis Figure 2). The epidermis (outermost) is a non-vascular layer, which has a
171 protective role as a barrier to penetration of chemicals to the underlying vascular dermis. The
172 healthy human epidermis comprises 4 layers (stratum corneum, stratum granulosum, stratum

spinosum and stratum basale) separated from the dermis by the basement membrane (Breitkreutz, et al. 2013). The barrier properties of the skin lie mainly within the stratum corneum (SC), which has about 16 layers and takes about two weeks to completely desquamate (Hoath and Leahy 2003). This highly hydrophobic layer is composed of differentiated non-nucleated cells, corneocytes, which are filled with keratins and embedded in the lipid domain. Percutaneous penetration of molecules through the SC occurs mainly via passive diffusion but may also occur via sweat glands and hair follicles directly to the dermis. Although little is known about the expression and function of influx transport proteins in human skin and their role in dermal uptake of xenobiotics, The role of organic anion transporting polypeptides (OATP) in mediating the active transport process of large organic cations via human keratinocytes was highlighted (Schiffer, et al. 2003). Chemical residues limited to the epidermis will be eliminated from the exposed skin by desquamation and willnot be available for systemic distribution (Aggarwal, et al. 2014).

Significance of dermal absorption as a pathway of human exposure to FRs.

Although several studies have highlighted the importance of indoor dust ingestion as a pathway for human exposure to various FRs, few reports have discussed human dermal exposure to such contaminants (Stapleton, et al. 2012; Stapleton, et al. 2008; Watkins, et al. 2011). Watkins *et al.* (Watkins, et al. 2011) reported a strong positive correlation between PBDE levels on hand wipes (assumed to result from hand contact with contaminated dust or flame-retarded products) and PBDE concentrations in serum from American adults. While concentrations of PBDEs in indoor dust were strongly correlated with those in hand wipes, and infrequent hand-washers had 3.3 times the levels of PBDEs in their handwipes than did frequent hand-washers; correlation could not be established directly between PBDE concentrations in indoor dust and their levels in serum (Watkins, et al. 2011). In a more

recent contribution, significant associations between concentrations of TCEP, TCIPP, TDCPP, HBCD, TBB and TBPH in children handwipes and house dust were observed (Stapleton, et al. 2014). Another recent study reported 2-3 times increase of median concentrations of penta-BDE, TBB, and TBPH in paired handwipe samples of 11 gymnasts after practice compared to before (Carignan, et al. 2013). This opens up the possibility that FRs in dust may also be an indicator of another exposure pathway, such as direct dermal uptake of FRs present in treated goods (e.g. games consoles, remote controls, and fabrics). A pivotal issue for risk assessment studies is the influence of indoor contamination with FRs on human body burdens. Understanding of this remains incomplete. One approach is that of Lorber (Lorber 2008) who used a simple pharmacokinetic (PK) model to predict the body burdens of PBDEs in American adults using intake data from different exposure pathways. Predicted body burden were compared with measured data and the relationship between external and internal exposure discussed. Since then, a few studies have applied similar PK models with slight adjustments to further understanding of the relationship between concentrations of PBDEs, HBCD and TBBP-A in the environment and human body burdens (Abdallah and Harrad 2011; Johnson-Restrepo and Kannan 2009; Trudel, et al. 2011). Further to identifying various research gaps including the bioavailability of FRs following ingestion of indoor dust and the elimination half-lives of these compounds in human, One major outcome of such PK studies is the highlighted potential importance of dermal contact with indoor dust and/or FR-containing items as a pathway of exposure to BFRs. To illustrate, dermal uptake was reported as the 2nd most important contributor(following dust ingestion) to PBDE body burdens of Americans. This was despite a very conservative assumption – *made in the absence of experimental data* - that only 3% of PBDEs with which dermal contact occurred (via adherence of indoor dust to the skin) were absorbed (Lorber 2008). Moreover, a recent PK model reported ingestion of diet and dust, as well as dermal exposure

to dust to constitute the major factors influencing human body burdens of PBDEs in both Americans and Europeans. Once again, these conclusions were founded on low assumed values of dermal absorption efficiency (2.5-4.8%) (Trudel, et al. 2011). Neither study considered potential dermal absorption following contact with FR-treated items and assumed percutaneous penetration fractions based on values reported for dermal absorption of dioxins and PCBs from soil in laboratory animal models (Lorber 2008; Trudel, et al. 2011). Boyce et al. (2009) applied a Monte Carlo-based mathematical approach for assessment of human exposure to TBBPA, DBDPE and BDE-209 via indoor dust ingestion and dermal contact. Based on physicochemical properties, analogy with data for PCBs and the absence of any chemical-specific studies, dermal absorption values of 10%, 0.1% and 1% were used for TBBPA, DBDPE and BDE-209, respectively. Results revealed dermal contact with indoor dust made significant contributions (15 - 40%) to estimates of overall human exposure to these BFRs in North America and Europe. The authors highlighted that at such significant contribution levels; inaccuracies in the dermal absorption factors applied could have dramatic effects on exposure assessments (Boyce, et al. 2009).

Transdermal metabolism of xenobiotics.

Besides the role of the stratum corneum as the major structure for epidermal barrier function, there is increasing evidence that xenobiotic metabolizing enzymes and transport proteins function as a second biochemical barrier of the skin (Esser and Goetz 2013; Gundert-Remy, et al. 2014; Wiegand, et al. 2014). Currently, very little is known about the transdermal metabolism of flame retardant chemicals. Garner and Matthews confirmed extrahepatic dermal metabolism of mono- to hexa- PCBs in F-344 male rats. However, the exact chemical structure of the formed metabolites was not confirmed (Garner, et al. 2006). Another *in vitro* study reported the dermal metabolism of BDE-209 and TDCPP to be minimal in adult female

mice (Hughes, et al. 2001). However, an extensive literature exists on the capacity of human skin to metabolise various chemical compounds. Recent findings indicate that human skin possesses not only multiple cytochrome P450 isoenzymes, but also influx and efflux transporter proteins. While the pattern of cytochrome P450 isoenzymes in the skin differs from the pattern in the liver, It seems likely that the skin can participate in both Phase I (e.g. oxidation, reduction and hydrolysis) and Phase II (e.g. glucuronidation and acetylation) metabolic reactions (Gundert-Remy, et al. 2014; Merk 2009). Moreover, human skin cells contained at least five different esterases reported to act on simple ester bonds in organophosphate compounds (paraoxon and bis(4-nitrophenyl)phosphate). Therefore, dermal biotransformation may play an important role in the ultimate fate and bioavailabilty of FRs in the skin, especially for PFRs and NBFRs which have labile functional groups.

In vivo dermal bioavailability studies

While the most reliable method for assessment of dermal absorption for human risk assessment would involve study of human volunteers; technical and ethical constraints means their use has been and will likely remain limited (Jakasa and Kezic 2008). Although the use of *in vivo* animal models has been strongly discouraged (European Commission and absorption 2004; Howes, et al. 1996), their application for dermal risk assessment is of value because they represent an intact physiological and metabolic system when the use of human volunteers is not possible. Furthermore, *in vivo* animal models (especially rats) have long been used by different industrial and regulatory institutions to provide data on various toxicokinetic and toxicodynamic parameters, as well as dermal absorption (Zendzian 2000). While dermal uptake of environmental contaminants (e,g, polycyclic aromatic hydrocarbons, phthalates and pesticides) from soil and sediment has been reviewed (Spalt, et al. 2009), very little is known about the uptake of flame retardants via skin (Table 1). Schmid et al. studied

the dermal absorption of PCBs in one human volunteer (52 year old male, 65 kg body weight) (Schmid, et al. 1992). The volunteer was exposed to a mixture of 8 tetra- to hepta-^{13}C-PCBs for different time spans using cotton cloth and aluminium foil as carrier materials to mimic real life situations of skin contact with PCB-contaminated clothes or metal surfaces. After exposure the skin was washed subsequently with water and ethanol. Non-absorbed ^{13}C-PCBs were determined in the washing solvents and in the carrier materials, while the bioavailable fraction was measured in plasma samples collected at 0.5-6 days post-exposure. Results revealed low percutaneous absorption (PA) of target PCBs equivalent to 6 % of the absorption after oral intake of the same amount. The absorption rate was largely dependent on the site of administration, on the carrier material (higher from the aluminium foil than the cotton cloth) and almost not on the amount administered where the percentage uptake remained constant at long (8 hours) and short (10 min) exposure times(Schmid, et al. 1992). Similar PA values (3.4-4.5 %) were reported in Rhesus monkeys exposed to PCB-contaminated soil for 24 h (Mayes, et al. 2002). The difference between the calculated PA values for soil PCBs in this study and the 14% dermal absorption factor used by the USEPA (U.S. EPA 1992) was attributed mainly to soil organic content in addition to particle size, skin residence time and contaminant "aging" in the soil. The percutaneous absorption of ^{14}C-Aroclor 1260 in test monkeys was determined by measuring the radioactivity in excreta (equation 1) (Mayes, et al. 2002).

$$\%\ \text{Dose Absorbed} = \left(\frac{\%\ \text{Topical Dose Excreted}_{(^{14}\text{C-urine} + {}^{14}\text{C feces})}}{\%\ \text{Intravenous Dose Excreted}_{(^{14}\text{C-urine} + {}^{14}\text{C feces})}} \right) \times 100 \quad \text{....(1)}$$

An important point is that the model used in equation 1 and in all *in vivo* studies in humans or surrogate species where the animal is not sacrificed, cannot account for any test compounds sequestered within the skin (Mayes, et al. 2002; Spalt, et al. 2009). This may lead to substantial underestimation of the actual dermal uptake of persistent lipophilic compounds which would eventually (within days) be systemically absorbed from the skin depot of the

298 exposed organism. For such compounds, for which the outcome of concern is typically not
299 acute toxicity, inclusion of skin burden is necessary (Spalt, et al. 2009). While adjustment for
300 excretion following intravenous administration may be employed, this has associated
301 uncertainty and presumes no difference in the excretory pattern associated with dermal and
302 intravenous administration used as a reference. The importance of this concept of
303 contaminant skin depot was confirmed by Garner and Mathews (Garner and Matthews 1998).
304 These authors applied 0.4 mg/kg body weight of a mixture of radiolabeled mono- to hexa-
305 PCBs in acetone to a 1 cm^2 hairless skin area at the back of adult male F-344 rats.
306 Distribution of radioactivity in the dose site and selected tissues was determined by serial
307 sacrifice at time points up to 2 weeks. Results revealed the dermal penetration of test
308 compounds to vary inversely with degree of chlorination and at 48 h ranged from ca. 100%
309 for mono-PCB to ca. 30% for hexa-PCB. Although the maximum internal exposure to Mono-
310 PCB was at 4 h (37% of the dose present in tissues), only 0.2% of the absorbed dose
311 remained in the tissues after 2 weeks. In contrast, tetra-PCB internal exposure was the
312 greatest with ca. 85% of the total absorbed dose present in tissues 72 h postadministration.
313 Furthermore, hexa-PCB equivalents in tissues continued to rise through 2 weeks postdose
314 (~15% of absorbed dose) since systemic absorption from epidermis depots was still
315 incomplete when the study was terminated. While rat skin favoured the rapid absorption of
316 lower chlorinated PCBs; their relatively rapid metabolism and elimination, suggests lower
317 body burdens of the less chlorinated congeners compared to higher molecular weight PCBs
318 which penetrate less rapidly, but persist at the site of exposure and slowly enter the systemic
319 circulation (Garner and Matthews 1998). In another contribution, Garner et al (Garner, et al.
320 2006) used the same animal model to study the disposition of mono- to hexa- PCBs following
321 dermal administration. Results confirmed higher chlorinated PCBs to be slowly absorbed and
322 accumulated in the adipose and skin. Interestingly, excretion and metabolic profiles following

dermal dosing tended to differ from profiles following equivalent intravenous doses. This was attributed to first pass metabolism occurring at the dermal dose site. The study further suggested that the rate of absorption, and consequently disposition of PCBs following dermal exposure, may be mediated, either in part or fully, by transdermal metabolism (Garner, et al. 2006).

The dermal absorption of the flame retardant resorcinol bis-diphenylphosphate (RDP) was investigated in rats and monkeys. Sprague-Dawley rats and cynomolgus monkeys were dermally exposed to 100 mg of ^{14}C-RDP spread over a shaved area representing about 20% of the animal's surface area. Results revealed ~ 20% of the dermal dose was absorbed in rats, whereas primates absorbed only 10% of the applied dermal dose (Freudenthal, et al. 2000).Very little is known about the dermal absorption of BFRs. In an early report, Ulsamer et al. studied the dermal absorption of the banned flame retardant tris (dibromopropyl) phosphate (TRIS) in rabbits. The test animals were exposed to radiolabelled ^{14}C-TRIS via sections of fabric (10 x 12 cm) placed in contact with skin for 96 h. Results revealed that up to 17% of the applied dose was absorbed when the fabric was wetted with urine. Only 6% of the dose was absorbed when the cloth was wetted with simulated sweat, which was slightly higher than the absorption (4%) from a dry cloth (Ulsamer, et al. 1978). A more recent study used a female C57BL/6 mice model to assess the dermal bioavailability of BDE-47. Test animals were exposed to 1 mg/kg body weight of ^{14}C-BDE 47 in acetone applied to a hairless 2 cm^2 skin patch. Results revealed ~62% absorption of the administered dose after 5 days while 15% remained at the site of application where skin and adipose were reported as the major depot tissues (Staskal, et al. 2005).

Paradigm shift – *in vivo* to *in vitro* dermal bioavailability studies

Due to the ethical and technical issues arising from the use of lab animals in toxicology studies, the use of *in vivo* animal models is increasingly strongly discouraged (Jakasa and Kezic 2008). Therefore, focus has shifted to developing and validating alternative *in vitro* test methods, which also provide a better platform for development of predictive pharmacokinetic models. Several guidance documents for conducting *in vitro* skin absorption studies (OECD 2004; U.S. EPA 2004; WHO 2006) are currently available rendering the application of *in vitro* skin models increasingly acceptable for research and regulatory purposes.

Different types of skin may be used, for example, human excised skin from surgery or from cadavers (*ex vivo* skin) or animal (e.g. pig) skin. Various types of diffusion cells have been employed in *in vitro* studies to date, and the composition of receptor fluids may vary. All these factors can influence the results of *in vitro* experiments (Jakasa and Kezic 2008). While several papers have reported on *in vitro* dermal absorption of environmental contaminants such as: polycyclic aromatic hydrocarbons, phthalates, as well as organochlorine and organophosphate pesticides (Hopf, et al. 2014; Hughes and Edwards 2010; Spalt, et al. 2009); very few *in vitro* studies of the dermal absorption of FRs exist. In one such study, Hughes et al. (Hughes, et al. 2001) used skin from adult hairless female mice (SKH1) mounted in flow-through diffusion cells to study the absorption of ^{14}C-BDE-209 and ^{14}C-TDCPP at 3 concentration levels. HEPES ((*4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid*))-buffered Hanks' balanced salt solution (pH 7.4) with 10% fetal bovine serum was used as receptor fluid. Following 24 h exposure, the skin patches were washed with solvent prior to analysis of receptor fluid, skin wash and skin for chemical-derived radioactivity. BDE-209 showed low penetration (0.3%) into the receptor fluid while up to 20% of the dose remained in skin after 24 h. TDCPP displayed higher penetration (39–57%) to the receptor fluid, while 28–35% of administered dose remained in the skin. This was mainly attributed to its lower molecular weight and K_{OW} than BDE-209 (Hughes, et al. 2001). The dermal absorption of

BDE-47 was studied using *in vitro* split-thickness skin membranes (350–410 μm, stratum corneum uppermost) of human and rat skin exposed to a single dose of ca. 10 mg/cm^2 of ^{14}C-BDE-47 for 24 h. The skin patches were mounted in flow-through cells while receptor fluid (NaCl, 0.9%, w/v in water) was pumped through the receptor chambers at ca. 1.5 ml/h (Roper, et al. 2006). The dose recovered from the receptor fluid was 2% and 15% of administered BDE-47 to human and rat skin, respectively. The difference between the results of this *in vitro* study (Roper, et al. 2006) and the higher (62%) sorption observed in an *in vivo* study of dermal absorption in mice (Staskal, et al. 2005) (Table 1) may be attributed mainly to the use of 0.9% NaCl solution in water as a receptor fluid, as this may greatly reduce diffusion of the lipophilic BDE-47 to the receptor fluid (Wilkinson and Williams 2002) and does not accurately mimic actual biological conditions. Possible evidence of this is provided by the high residual levels of BDE-47 detected in the cells (57% and 33% for human and rat skin, respectively) that appeared not to diffuse to the receptor fluid (Roper, et al. 2006). While no data exists on dermal absorption of TBBP-A, a recent *in vitro* study reported on the percutaneous bioavailability of its precursor, bisphenol A (BPA) from human and pig skin (Zalko, et al. 2011). Viable human and pig skin patches (500 μm thickness) were maintained at the air/liquid interface using Transwell inserts while dermal/epidermal feeding was achieved via diffusion of nutrients from a modified Dulbecco's Eagle culture medium which kept the cells alive during 72 h exposure experiments. BPA was efficiently absorbed (65% and 46% from pig and human skin, respectively) and metabolised by the cultured skin indicating the trans-dermal route contributes substantially to human exposure to BPA (Zalko, et al. 2011). However, it should be noted that TBBP-A has a much higher molecular weight and consequently, different physico-chemical properties (e.g. water solubility, partition co-efficient and vapour pressure) than BPA. Furthermore, the lack of halogen atoms in BPA is

likely to enhance the rate of its percutaneous absorption compared to its tetra-brominated derivative (Garner and Matthews 1998).

Given the growing evidence that suggest dermal absorption to be a potentially significant pathway of human exposure to FRs, the paucity of data on dermal bioavailability of such ubiquitous contaminants may be attributed to a combination of ethical, technical and economic issues. One alternative method with the potential to overcome such difficulties is the use of 3D human skin equivalent (HSE) models which provide a relatively cheap, commercially available, ethical, and reliable method for dermal absorption studies that is capable of producing data of relevance to human exposure.

Human Skin Equivalent models (HSE)

Rationale. Although the Organisation for Economic Co-operation and Development (OECD) and the European Centre for Validation of Alternative Methods (ECVAM) describe methods for assessing dermal absorption using excised *in vitro* human and animal skin, the lack of correlation in transdermal permeation of chemicals across species imparts a high degree of uncertainty when extrapolating results from animal models to humans. This is mainly due to variations in the stratum corneum thickness, intercellular subcutaneous lipids and/or between-species differences in metabolic enzymes and their activity (Schafer-Korting, et al. 2008a). Therefore, excised *in vitro* human skin is preferable to animal skin (e.g. rat or pig skin) for dermal absorption testing, but is clearly less available. To overcome this shortage, HSE have been developed to provide an alternative to human skin in testing of compounds for transdermal permeability (Mertsching, et al. 2008). A protocol was developed and validated according to the OECD guidelines for percutaneous absorption by using commercially available HSE models (Table 2). The permeability of tested HSE models were compared to that of excised human epidermis, pig skin and bovine udder skin, using 9 compounds widely varying in physicochemical characteristics, including the OECD standards: testosterone,

caffeine and benzoic acid. Results revealed HSE models closely mimic the histological and physiological character of viable human skin, allowing their use for *in vitro* skin penetration studies, taking product-specific overpredictability into account (Hartung, et al. 2004; Schafer-Korting, et al. 2008a). Consequently, several validated methods using HSE models have been approved by OECD and ECVAM for testing skin absorption, phototoxicity, corrosion and irritation by xenobiotic chemicals (Ackermann, et al. 2010; Buist, et al. 2010).

Composition. HSE models can be generally classified into 2 main types:

1- Reconstructed Human Epidermis (RHE): RHE is a human skin tissue obtained from human keratinocytes cultured on an inert polycarbonate medium. One key advantage is that it permits growth of donor epidermal cells in a serum-free culture environment. After rapidly proliferating preparative keratinocyte cultures have been obtained, the epidermal cells yielded are seeded on inert filter substrates, which are then raised to the air-liquid interface in a humidified-air incubator. A fully-defined nutrient medium feeds the basal cells through the filter substratum. After 14 days, a stratified epidermis is formed that closely resembles human epidermis *in vivo* (Figure 3) (Boelsma, et al. 2000).

Morphologically, these cultures exhibit a well-stratified epithelium and cornified epidermis with significantly improved barrier function and metabolic activity (Boelsma, et al. 2000). Differentiation markers such as suprabasal keratins, integrin b4, integrin a6, fibronectin, involucrin, filaggrin, trichohyalin, type I, III, IV, V and VII collagen, laminin, heparan sulfate and membrane-bound transglutaminase are expressed similar to those of the human epidermis (Brinkmann, et al. 2013; Mehul, et al. 2004).

Several RHE models are now commercially available. The different models share the air-exposed culture conditions, but differ in the support used as a dermal equivalent on which the human keratinocytes are grown (Table 2). Numerous histological and biochemical features are shared by these models, in particular epidermal stratification and differentiation, and all

446 produce a well-defined stratum corneum as a result of tightly regulated expression of
447 differentiation-related genes (Boelsma, et al. 2000; Zhang and Michniak-Kohn 2012).

448 *2- Full-Thickness skin (FT):* Paracrine signaling between dermal fibroblasts (FB) and
449 epidermal keratinocytes (KC) is believed to modulate skin responses during contact irritant or
450 allergic reactions. Dermal FB also play an important role in photo-aging, photo-damage,
451 wound healing and cancer progression. To enable *in vitro* investigation of these and other
452 dermal phenomena in which FB-KC interactions are important, FT skin models composed of
453 a FB-containing dermis/KC-containing epidermis have been developed (Schafer-Korting, et
454 al. 2008b; Semlin, et al. 2011). In order to test possible immunological reactions on skin,
455 Langerhans cells (LCs) can be introduced into FT skin substitutes (Regnier, et al. 1997).
456 Percutaneous absorption of chemicals is due to two different routes of passive diffusion. The
457 first is trans-epidermal diffusion via inter- or trans- cellular pathway across the stratum
458 corneum, whereas the second is trans-appendageal diffusion via hair follicles and associated
459 sebaceous glands. The presence of appendages in the FT models may represent another
460 advantage added to their superiority over RHE models for biotransformation-linked toxic
461 endpoints (Ackermann, et al. 2010; Curren, et al. 2006). However, the scarce information
462 available to date, indicates a complex relationship between percutaneous absorption, skin
463 thickness and lipophilicity of test compounds (Wilkinson, et al. 2006). This is further
464 compounded by factors like: exposure vehicle, diffusion cell design and receptor fluid
465 (Schafer-Korting, et al. 2008b).

466 ***General Protocol for in vitro percutaneous absorption studies.*** Each HSE model is supplied
467 with its respective receptor/culture fluid and its percutaneous absorption protocol. Generally,
468 the protocol involves mounting the fully-developed skin patches at the air-liquid interface of
469 a permeation device (e.g. Franz-cell type diffusion cells, Mattek® permeation device, see SI
470 section for further details) while in contact with the receptor fluid. The test compound is then

applied to the surface of the stratum corneum and incubated for the required exposure time (usually 24 h). The receptor fluid is sampled and replaced at fixed time intervals. At the end of the exposure period, the skin surface is washed/wiped clean of any residual contaminant remaining, prior to collection of the receptor fluid and cell culture for chemical analysis (Figure 4).

Future perspectives and challenges facing dermal absorption studies of FRs

Although current commercially available HSE models may provide a useful alternative to study the human dermal absorption of FRs, there remains several challenges and research gaps that need to be addressed in the near future. These include:

- The lack of experimental data –either *in vivo* or *in vitro*– relevant to the dermal bioavailability of a wide range of FRs in human. Such paucity of information regarding the dermal pathway hinders the current efforts for accurate risk assessment of various FRs. Furthermore, it complicates the pharmacokinetic modelling studies aiming to understand the relationship between external exposure and human body burdens of FRs.
- The diverse nature and wide range of physico-chemical parameters of organic FR chemicals (Table SI-1). Contaminant properties like: Log K_{OW}, molecular weight, size and water solubility were reported to affect the dermal absorption of PCBs (Garner and Matthews 1998). Furthermore, the difference in protein binding affinities of various FRs may also influence their permeation through the skin barrier. This will be of particular interest if OATPs were involved in mediating the active transport process of FRs across the human epidermis. Therefore, the chemical diversity and co-existence of various BFRs and PFRs in different environmental samples are likely to present a challenge to environmental scientists trying to mimic *in vivo* scenarios.

- FR chemicals with similar/comparable molecular weight, size and Kow can exist in different isomeric forms (e.g. HBCD isomers), which might adopt various structural characteristics (e.g. planarity) and exhibit different physico-chemical properties (e.g. water solubility). This is also likely to constitute an important factor influencing the dermal bioavailabilty of such iso-baric compounds.
- Despite the huge advances in production and validation of HSE models in the past few years, further improvements are still required to closely mimic the *in vivo* situation. The presence of hair follicles, sweat and sebaceous glands provides further potential pathways for percutaneous penetration. The dermis *in vivo* is continously perfused by the subcutaneous vasculature, which can rapidly remove permeants reaching the epidermal-dermis interface, allowing for further diffusion of the permeant through the skin layers. This system can be mimicked *in vitro* via the use of dynamic in-line flow through diffusion cells (Table SI-2). However, further validation and standardisation of test protocols using this model is still required to gain the approval of the regulatory bodies and research organisations.
- Transdermal metabolism has been reported as a major mediator for percutaneous absorption of PCBs (Garner, et al. 2006). Currently, very little is known about the dermal biotransformation of BFRs and PFRs (Hughes, et al. 2001). Enhanced understanding of percutaneous metabolic pathways and identification of the metabolites thus formed in humans thus appears important, if the reliability of risk assessment of these contaminants is to be improved.
- The excretion of xenobiotic chemicals and their metabolites in sweat and hair follicles has been well documentd in literature (De Giovanni and Fucci 2013; Parle and Jadhav 2007). Therefore, biotransformation may not be the only dermal contaminant-removal mechanism in human. Further research is required to understand the role of eccrine

sweat and hair follicles as excretion routes for FRs. Consequently, the *in vitro* human skin models may consider the dermal bioavailability of FRs as an equilibrium process.

- While HSE models have been widely exploited in the pharmaceutical and cosmetic fields; to the authors' knowledge, they are yet to be applied for studying dermal absorption of FRs or any other organic contaminants. This is likely to create several challenges for analytical method development, exposure protocols and modelling of the results. Furthermore, *in vitro* dermal studies carried out for the purpose of risk assessment should also include scenarios that mimic real life exposure to the test compounds. This includes exposure to environmentally-relevant concentrations via appropriate exposure media. Previous studies have shown that dermal absorption of PCBs from contaminated soils was different from direct application of PCBs in solution to the skin (Mayes, et al. 2002). In addition, dermal bioavailability has also been shown as influenced by the age of the contaminant in soil and its organic content (Spalt, et al. 2009). Similar factors are likely to affect percutaneous absorption of BFRs and PFRs. Therefore, several exposure scenarios addressing dermal uptake from a range of environmental media (e.g. indoor dust, soil, sweat and consumer products) will be needed for full characterisation of the exposure arising from human dermal exposure to FRs.

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Supplementary data

Specific details on physico-chemical parameters, uses, toxicokinetic profiles and main exposure pathways of key brominated and phosphorous flame retardants in addition to different *in vitro* dermal absorption protocols are available as supplementary data.

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Tables

Table 1: Summary of *in vivo* and *in vitro* methods applied for studying dermal absorption of FR chemicals.

Compound	Skin type	Study type	Dosing	Exposure time	Absorption (% of administered dose)	Ref.
PCBs # 52, 101, 108, 118, 138, 153, 170, 180	Human	*In vivo*	PCBs (5 mg) were dissolved in DCM and applied to the carrier (4cm2 cotton cloth or 28cm2 aluminium foil) prior to fixing to the skin	0.66-1 day	Up to 6% for PCB-153	(Schmid, et al. 1992)
PCBs (^{14}C-Aroclor 1260 mixture)	Rhesus monkeys	*In vivo*	500 mg of 70 µg/g PCB-spiked soil applied to 12 cm^2 of skin	12-24 h	3.43 ± 0.35% for 12 h and 4.26 ± 0.52% for 24 h	(Mayes, et al. 2002)
PCBs # 4, 15, 47, 155	Male F-344 rats	*In vivo*	0.4 mg/kg bw applied to 1 cm^2 of skin	1, 4, 8, 12, 24, 48, 72, 96, and 336 h	From ca. 100% for PCB-4 to ca. 30% for PCB-155.	(Garner and Matthews 1998)
BDE-47	Female C57BL/6 mice	*In vivo*	1 mg/kg bw applied to 2 cm^2 of skin	5 days	62%	(Staskal, et al. 2005)

BDE-209 and TDCPP	female mice (SKH1)	*In vitro*	6, 30 and 60 nmol in THF for BDE-209; 20, 100 and 200 pmol in acetone for TDCPP	24 hrs	2–20% in skin, 0.07-0.34% in receptor fluid for BDE-209. 39–57% in skin and 28–35% in receptor fluid for TDCPP	(Hughes, et al. 2001)
BDE-47	Human and rat skin (350–410 µm)	*In vitro*	10 mg/cm^2 applied in acetone.	24 hrs	2-15% in 0.9% NaCl receptor fluid; 57% and 33% remained in cells for human and rat skin, respectively.	(Roper, et al. 2006)
BISPHENOL-A (Precursor to TBBP-A)	Pig Ear Skin and Human skin	*In vitro*	50, 100, 200, 400 and 800 nmol were applied in 60 µL ethanol/phosphate buffer (pH 7.4)	24, 48 and 72 h	Human skin (45.6 ± 6.2%), pig skin (65.3 ± 8.2%) BPA–glucuronide formed in human skin , corresponding to 7 ± 2, 16 ± 3 and 30.± 3 nmol at 24, 48 and 72 h, respectively.	(Zalko, et al. 2011)

872 **Table 2: Characteristics of commercially available HSE models.**

Brand Name	Scaffold material	Source	Dermis	Manufacturer
Episkin™	Collagen (0.38 cm^2)	Keratinocytes(Mammary/Abdominal samples obtained from healthy consenting Donors during plastic surgery)	NO	L'Oreal, Nice,France
Skinethic™	Polycarbonate membrane (0.5 cm^2)	Keratinocytes (neonatal foreskin tissue or adult breast tissue)	No	L'Oreal, Nice,France
Epiderm™	Collagen coated Polycarbonate (9mm diameter)	Human keratinocytes (neonatal foreskin adult breast skin)	No	MatTek Corporation, MA, USA
EpidermFT™	Collagen	Human keratinocytes (neonatal foreskin adult breast skin) human fibroblasts (neonatal skin, adult skin)	Yes	MatTek Corporation, MA, USA
EST-1000	Polycarbonate membrane	Keratinocytes (neonatal foreskin)	No	CellSystems, Troisdorf Germany
AST-2000	Collagen	Human Keratinocytes	Yes	CellSystems, Troisdorf Germany
Phenion® FT Model	Bovine, cross linked,lyophilized collagen (1.3 cm dia)	Primary human keratinocytes (neonatal foreskin), human fibroblasts (neonatal foreskin)	Yes	Henkel, Duesseldorf, Germany
StrataTest®	Collagen I (0.6 cm^2)	immortalized, human NIKS® keratinocytes dermal fibroblasts	Yes	Stratatech Corporation Madison WI, USA
Epistem ® LSE	Collagen	Primary human keratinocytes and dermal fibroblasts.	Yes	Epistem limited, Manchester, UK.
StratiCell® EPI/001	Polycarbonate membrane	Primary human keratinocytes	No	Straticell Corporation, Gembloux, Belgium.
StratiCell® Mel/001	Polycarbonate membrane	Primary human keratinocytes and melanocytes.	No	Straticell Corporation, Gembloux, Belgium.

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Figures

Figure 1: Major pathways of human exposure to FRs.

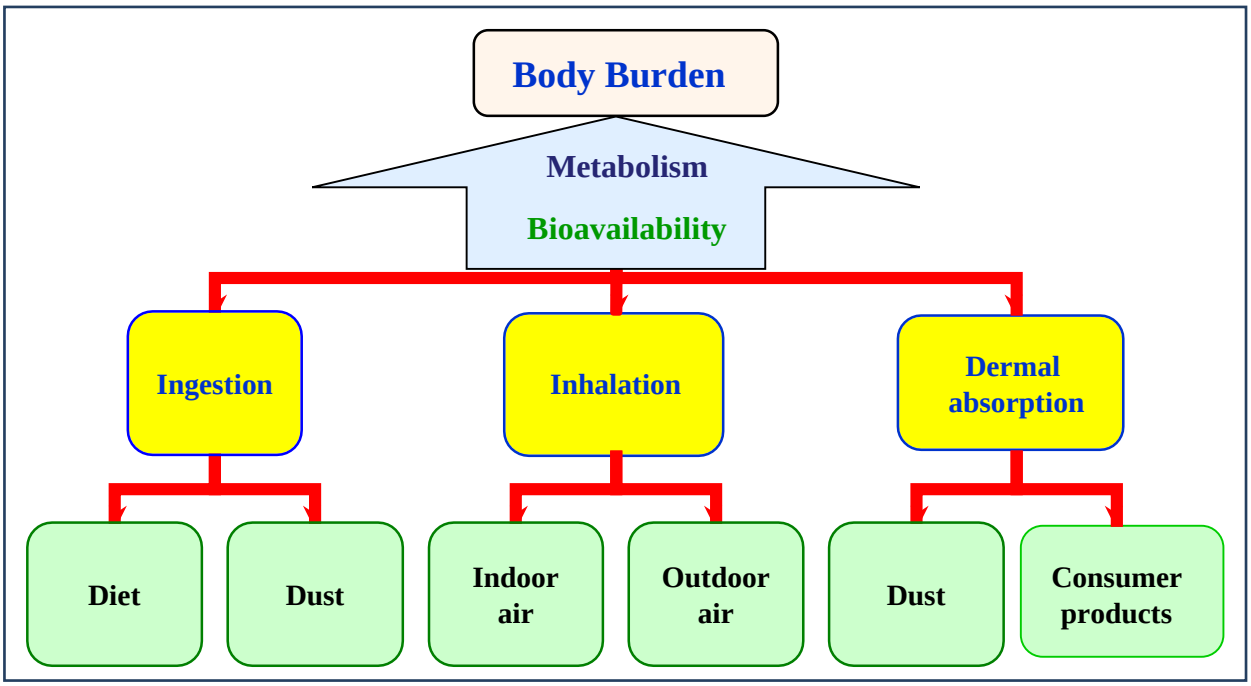


Figure 2: Anatomy of the human skin.

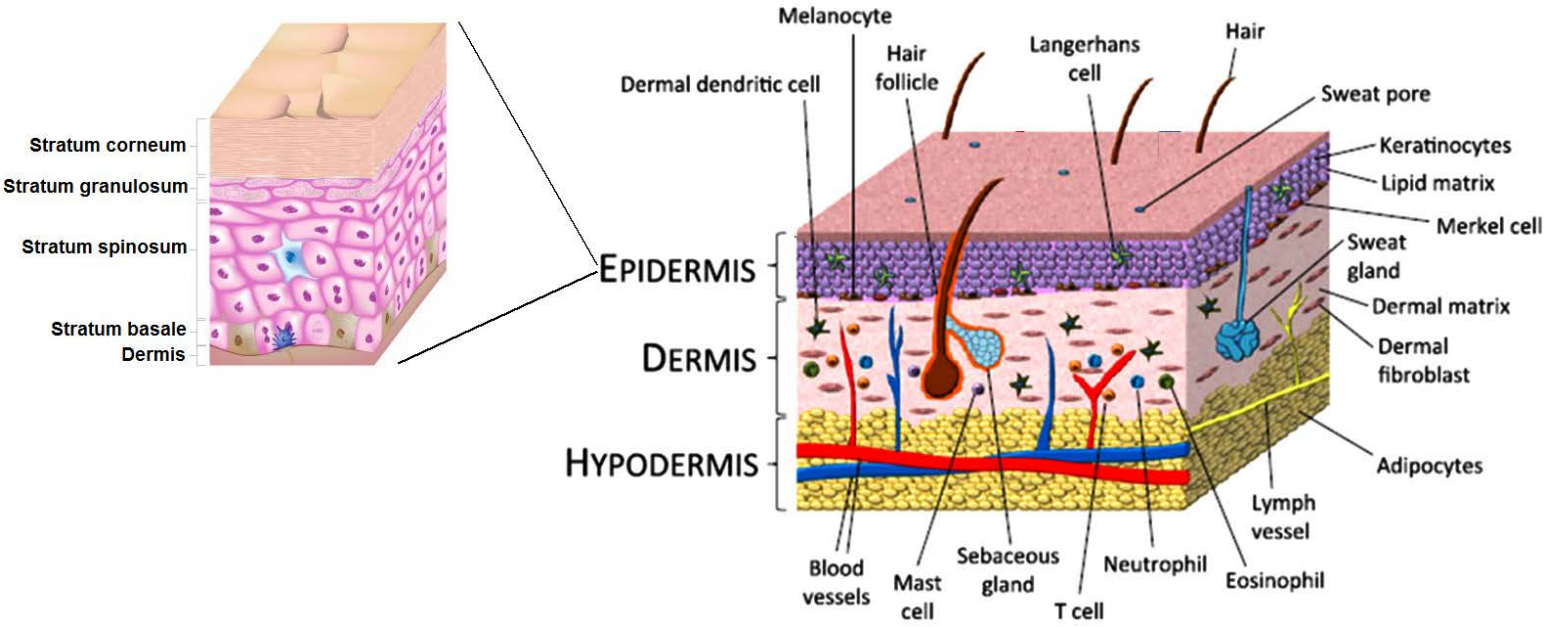
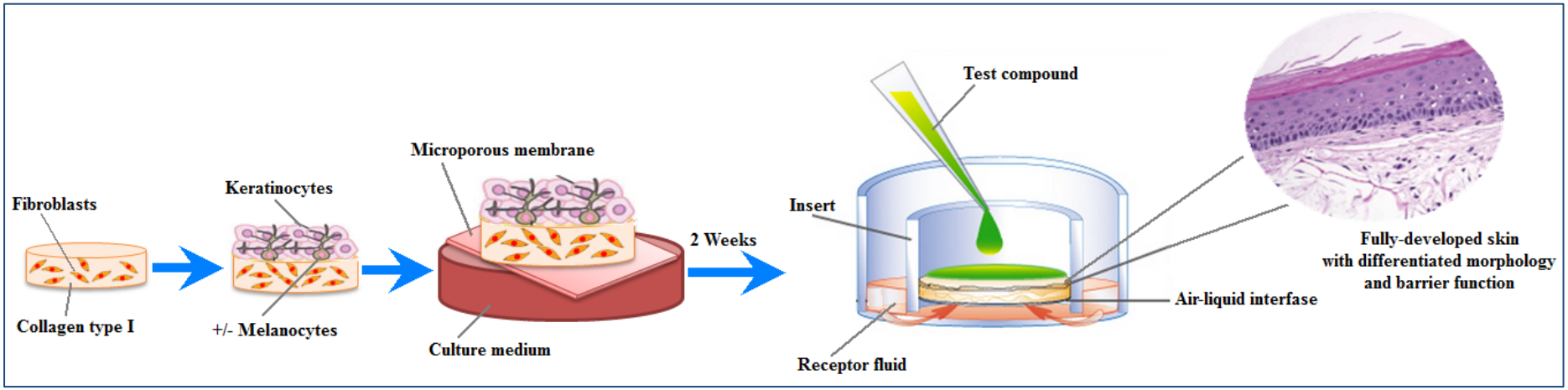


Figure 3: General stages of development of HSE model.



910 **Figure 4: General protocol for percutaneous absorption studies using *in vitro* HSE**

911 **models.**

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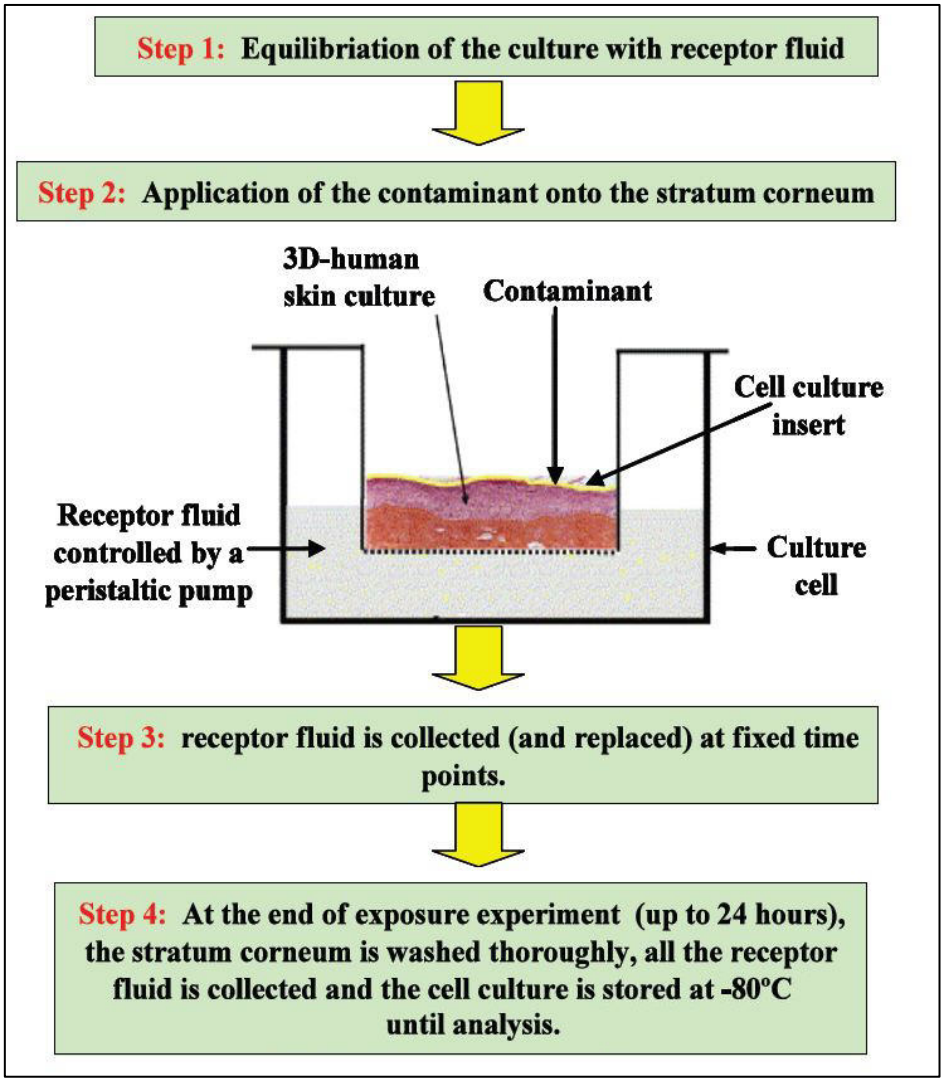

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